On page 47, line 13, following "See Figure" delete "1D" and substitute therefor ---1C---.

On page 48, line 16, insert a period immediately following "Natl".

On page 56, line 27, within the parentheses, delete "precipitable" and substitute therefor ---soluble---.

On page 75, line 5, delete "ß93Cys" and substitute therefor ---β93Cys---.

In the Drawings

In FIG. 16, in the label for the vertical axis, immediately preceding "Absorbance at 418 nm" insert ---A---.

N.C. as Amended Fig 16

REMARKS

The specification has been amended to correct minor, inadvertant errors. Support for the amendment to FIG. 16 can be found on page 75, lines 17-19.

At page 56, line 27, the word *precipitable* has been corrected to *soluble*, as it is evident to one of skill in the art that *soluble* is the intended word that provides consistency with the properties of low molecular weight molecules. In many enzymatic assays in molecular biology, a low molecular weight molecule becomes covalently bound to a high molecular weight molecule, or a high molecular weight molecule is cleaved to release a low molecular weight molecule. The high and low molecular weight products can be separated by acid precipitation (such as by trichloroacetic acid), whereby a precipitated high molecular weight product can be collected on a filter and the low molecular weight product can be collected as the filtrate. Alternatively, the low molecular weight product can be collected in the supernatant after centrifugation to pellet insoluble (precipitated high molecular weight) material. See, for an

example of an assay employing trichloracetic acid precipitation, Ausubel, F.M., *Current Protocols in Molecular Biology*, John Wiley & Sons, Inc., containing supplements up through Supplement 40, January, 1998, pp. 18.7.4-18.7.5, especially "Support Protocol 2" on pages 18.7.14 and 18.7.15 (Exhibit 1).

It is apparent to one of ordinary skill in the art that what was meant here was that the assay was for low molecular weight thiols (high molecular weight thiols do not cross the cell membrane) and that the assay for these low molecular weight thiols involved separation from high molecular weight components by trichloroacetic acid precipitation of the high molecular weight molecules and recovery and assay of the low molecular weight molecules in a supernatant.

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Assay Conditions

The assay conditions can have profound effects on any enzyme activity: if pepsin is assayed at pH 7, it is essentially inactive, but at pH 3, it has good protease activity. Similarly T4 DNA ligase is inhibited by salt concentrations >150 mM. For kinases, it is a good idea to use preliminary experiments to identify the effects of pH, salt concentration, concentrations of cations such as Mg^{2+} or Mn^{2+}, and temperature to optimize the assay conditions for the kinase of interest. It is often advantageous to perform kinase assays at 30°C rather than 37°C because the lower temperature makes it easier to stay within the linear range of the kinase, thus providing more control of the assay.

Another factor to be considered when optimizing an assay for a newly identified kinase is the concentration of ATP in the reaction mixture. In these protocols and in general, detection of kinase activity is based on the transfer of radiolabeled phosphate from ATP to the substrate, so the concentration of ATP can be varied only a little. Also, the specific activity of the [γ-^{32}P]ATP must be known in order to actually measure phosphotransfer. Most kinases have a K_m for ATP of 1 to 100 μM. If there is too much ATP in the reaction mixture, it will be difficult to measure phosphotransfer. An ATP concentration of 50 to 100 μM tends to work well. At that concentration, the enzyme should be working at ≥50% of maximum, depending on its apparent K_m for ATP, and addition of sufficient [γ-^{32}P]ATP to measure phosphotransfer is not prohibitively expensive. Usually the substrate concentration is high so that the enzyme is working at or close to V_{max}.

Controls

Including the correct controls for a kinase assay is critical to the success of the assay, especially when the enzyme source is a cell or tissue extract. The appropriate controls should always include a no substrate control, a no enzyme source control, a heat-denatured enzyme control, and, for enzymes that require an activator or cofactor, controls that use irrelevant activator or cofactor and controls without activator or cofactor.

CAUTION: These assays use [γ-^{32}P]ATP which should be handled and disposed of according to safety regulations. See *APPENDIX 1F* and the institutional Radiation Safety Office for guidelines for proper handling and disposal of ^{32}P.

BASIC PROTOCOL 1

ASSAY FOR CYCLIC NUCLEOTIDE–DEPENDENT PROTEIN KINASES

Cyclic adenosine monophosphate (cAMP)–and cyclic guanosine monophosphate (cGMP)–dependent protein kinases (PrKs) are quite similar in structure and require similar assay conditions. cAMP-PrK is a heterotetramer consisting of two regulatory (R) subunits and two catalytic (C) subunits, R_2C_2. In each case the regulatory subunits have binding sites for two molecules of cAMP. When the cAMP concentration is elevated in cells after activation of receptor-linked adenylyl cyclases, cAMP binds to the R subunits, causing the affinity of the C subunits for R subunits to drop by about four orders of magnitude. The formation of R_2cAMP_4 dimers and free C-subunits is favored, and the C-subunits are now active.

The case of cGMP-PrK is slightly different in that there are no free catalytic subunits released. cGMP-PrK is composed of two identical subunits. Each subunit has a regulatory domain and a catalytic domain, which are homologous to the R and C subunits of cAMP-PrK. On binding four molecules of cGMP the enzyme is activated, presumably by a conformational change.

The assay procedures for these two kinases are very similar and the same protein substrate can be used for each one as detailed below. More recently, both of these kinases have been assayed using peptide substrates.

Materials

5× cyclic nucleotide–dependent kinase reaction buffer (see recipe)
10 mg/ml histone 2B in H_2O
[γ-^{32}P]ATP solution (see recipe)
20× cyclic nucleotide solution: 20 µM cyclic AMP in H_2O/20 µM cyclic GMP in H_2O
Enzyme sample containing cyclic nucleotide–dependent kinase activity (see Support Protocol 1), kept on ice until use
30° water bath

Additional reagents and equipment for TCA precipitation (see Support Protocol 2), adsorption onto P81 phosphocellulose paper (see Support Protocol 3), or electrophoretic analysis (see Support Protocol 4)

1. For each assay reaction, add the following to a 1.5-ml microcentrifuge tube kept on ice:

   4 µl 5× cyclic nucleotide–dependent kinase reaction buffer
   1 µl of 10 mg/ml histone 2B
   1 µl [γ-^{32}P]ATP solution (to give 5 µCi/µl and 5 µM ATP final)
   1 µl 20× cyclic nucleotide solution
   0 to 13 µl H_2O.

   Cap tube and warm the mixture in a 30°C water bath.

   Perform each assay in triplicate and include no substrate, no enzyme, and no cyclic nucleotide controls.

   The total reaction mix volume is 20 µl per reaction. The amount of water required depends on how much enzyme sample is used in step 2.

   Sufficient reaction mix can be prepared in a single tube for all the reactions by multiplying the quantities for a single reaction by the total number of reactions + 1. Then the total amount of reaction mixture (minus enzyme) for one reaction can be added to each tube. This is often very convenient and reduces the potential for pipetting errors.

2. Start the reaction by adding 1 to 14 µl ice-cold enzyme sample containing cyclic nucleotide–dependent kinase activity.

   The volume of enzyme used depends on the amount of activity in the enzyme sample. In a preliminary experiment, the maximum indicated amount can be used to gauge the extent of the phosphotransfer reaction. The volume can be reduced as appropriate in order to allow for linear incorporation of phosphate into the substrate during the assay.

   For enzyme samples that are immunoprecipitates adsorbed onto Sepharose beads (UNIT 10.16), prepare the reaction mix minus enzyme source first, warm it, then dispense it into the immunoprecipitate. For immunoprecipitates, it is advisable to scale up the reaction for a total volume of 100 µl: add 75 µl reaction mix to 25 µl immunoprecipitate bound to beads.

3. Incubate 10 min in a 30°C water bath.

4. Stop the reaction using the reagent appropriate for the analytic method to be used—20 µl ice-cold 10% TCA for TCA precipitation (see Support Protocol 2), or 10 µl or 20 µl ice-cold 2× SDS-PAGE sample buffer for electrophoretic analysis (see Support Protocol 4). Use 10 µl of the reaction mix for adsorption to P81 phosphocellulose paper (see Support Protocol 3). Proceed with analysis by one of those methods.

SUPPORT PROTOCOL 1

PREPARING A CELL LYSATE FOR KINASE ASSAYS

This protocol describes a method for detergent-induced cell lysis to prepare a crude extract containing kinase enzyme activity. Other methods of cell lysis may be appropriate (see *UNIT 12.1*). Hypotonic lysis and isolation of P100 and S100 fractions can also provide useful data on the recovery of a protein kinase as a soluble or membrane-bound activity.

Materials

Cultured cells: adherent cells at ~70% confluence in 100-mm tissue culture dishes or suspension cells at 10^6 cells/ml
PBS (*APPENDIX 2*), ice-cold
Lysis buffer (see recipe)
Protease inhibitor stock solutions (see recipe)
Microcentrifuge, 4°C

1. Wash the cultured cells twice in ice-cold PBS. Completely aspirate the final wash solution.
2. Add 0.75 ml lysis buffer to ~2×10^7 cells; for adherent cells check that the monolayer is completely covered. Add protease inhibitors from stock solutions to the appropriate final concentration: 1 mM PMSF, 100 μM benzamidine, 5 μg/ml leupeptin, 5 μg/ml pepstatin A, and 5 μg/ml antipain.

   A 0.75-μl lysate should provide sufficient material for immunoprecipitation (use 25% to 50% of the lysate for an initial experiment and scale up or down depending on the results) or simple purification. If the purification procedure involves more than one chromatography step, prepare a larger lysate because only 10% to 20% of the activity is recovered from each chromatography step.
3. Incubate the cells 10 min at 4°C. Scrape the cells from the dish and transfer the lysate to a labeled microcentrifuge tube.
4. Microcentrifuge the lysate 10 min at maximum speed, 4°C. Carefully remove the supernatant and transfer it to a clean microcentrifuge tube.

   Lysates should be used immediately until proper storage conditions (e.g., –70°C, liquid nitrogen) can determined experimentally. For some kinases, it may not be possible to store the lysate at all.

SUPPORT PROTOCOL 2

TCA PRECIPITATION TO DETERMINE INCORPORATION OF RADIOACTIVITY

One of the classical methods for separating a reaction product from the reactants is differential precipitation. In the case of protein kinase assays using a protein substrate and [γ-^{32}P]ATP, it is very easy to precipitate the protein and leave the [γ-^{32}P]ATP in the soluble fraction. Most proteins are quantitatively precipitated by trichloroacetic acid (TCA), so TCA can be used to precipitate proteins phosphorylated during a kinase assay. Protein precipitate can be captured by filtration and the filter can be washed with TCA. TCA precipitation is a quick and reproducible way to determine the extent of [^{32}P]phosphate transfer to protein substrates. However, peptides are not precipitated by TCA, so adsorption of labeled peptides to P81 phosphocellulose paper (see Support Protocol 3) is a more suitable method for analyzing the results of kinase assays that use a peptide as substrate.

Materials

Assay samples (see Basic Protocols 1 to 5 and Alternate Protocol)
5% and 10% (w/v) trichloroacetic acid (TCA), ice-cold
95% ethanol
Diethyl ether

Whatman GF-C glass-fiber filters
Vacuum manifold (e.g., Fisher)
20-ml scintillation vials
Scintillation counter

1. Stop the reaction by adding 20 µl ice-cold 10% TCA. Mix thoroughly and incubate 10 min on ice to precipitate protein.
2. Pipet sample onto Whatman GF-C glass-fiber filter held in a vacuum manifold. Allow the sample to pass through the filter. Rinse the tube with 500 µl ice-cold 5% TCA. Add this wash to the appropriate filter.
3. Wash each filter with 5 ml ice-cold 5% TCA solution four times. Wash once with 10 ml of 95% ethanol, then three times with 10 ml diethyl ether. Allow each filter to dry for a few minutes.

   CAUTION: *Diethyl ether is extremely flammable and washes should be performed in a fume hood away from any flame or heat source. Ether washes should be disposed of in an appropriate manner.*
4. Place the dry filters in a 20-ml scintillation vial and count in a scintillation counter by detection of Cerenkov radiation.

   Alternatively, if dpm quantitation is desired, place dry filter in 10 ml scintillation fluid and count.

   The filter can also be counted by Cerenkov radiation after the washes with 95% ethanol. If the filters are to be counted in scintillant, however, the diethyl ether washes are essential, because TCA (which is removed by the ether) causes significant chemiluminescence when placed in scintillation fluid, making the counts produced by the scintillation counter meaningless.

ADSORPTION ONTO P81 PHOSPHOCELLULOSE PAPER

SUPPORT PROTOCOL 3

One of the main methods to separate [^{32}P]-labeled proteins or peptides from [γ-^{32}P]ATP after a protein kinase assay reaction is by adsorbing the protein or peptide to P81 phosphocellulose paper. P81 paper is an ion-exchange matrix with net negative charge at most pHs. At low pH (such as in 75 mM orthophosphoric acid, which is used to wash the paper in this protocol), the excess [γ-^{32}P]ATP left after a kinase assay will not bind. Under the same conditions, however, the phosphorylated peptide will bind to the P81 paper if it carries a net positive charge at low pH. In practice, this is usually achieved by tagging a peptide with two or three arginine or lysine residues at the N- or C-terminus. At the pH of 75 mM orthophosphoric acid the arginyl or lysyl side chains are positively charged and will bind to P81 paper. It is necessary, of course, that the activity of the kinase in question is not affected by the addition of basic residues to the substrate. Usually five or more residues are placed between the hydroxy-amino acid phosphorylation site and the basic residues required for binding to P81 paper.

When proteins are used as substrates for kinases, the net charge of the protein is also a consideration for adsorption to P81 paper. As for peptides, adsorption to P81 paper is more suitable for basic proteins than for acidic proteins. Under the conditions described in this protocol, histones bind very well to P81 paper because they are highly basic, but casein binds very poorly or not at all because it is a very acidic protein.